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EP-A- 0 200 362
EP-A- 0 201 184
EP-A- 0 310 229
WO-A-88/10315
WO-A-89/06700

Biochemicals for Molecular Biology, Boehringer Mannheim 1987, pp. 107-108

Enzyme Nomenclature, Academic Press, 1984, pp. 302-303

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Description

FIELD OF THE INVENTION

5 This invention relates to a process for amplifying a specific nucleic acid sequence.

BACKGROUND OF THE INVENTION

10 The detection of a specific nucleic acid sequence present in a sample by probing the sample with a complementary sequence of nucleic acids is a known diagnostic technique. Nucleic acids are highly specific in binding to complementary nucleic acids and are thus useful to determine whether a specific nucleic acid is present in a sample. One must know the sequence of the specific nucleic acid to be detected and then construct a probe having a complementary nucleic acid sequence to the specific nucleic acid sequence.

15 In this application, the phrase "specific nucleic acid sequence" means a single stranded or double stranded nucleic acid which one wishes to amplify; "sample" means a mixture containing nucleic acids; "sufficiently complementary" means that two nucleic acids, a primer and a template, are capable of specific interaction which allows efficient, primer-dependent and template-directed synthesis of DNA, under given conditions of ionic strength and temperature.

20 Since nucleic acid probes are highly specific, it is preferable in some situations to probe the nucleic acid sequence itself rather than the protein produced by the nucleic acid sequence. As a particular example, a diagnostic method based solely on protein detection would be unreliable for determining the presence of infectious particles of hepatitis B virus, due to the presence of significant levels of non-infectious antigen particles which lack the DNA genome. In another example, the various subtypes of 25 human papilloma virus found in either pre-cancerous or benign cervical tumors can be distinguished only by the use of nucleic acid probe hybridization. Also, the microbiology of AIDS makes it certain that an assay based on the presence of AIDS specific nucleic acid sequence would be superior as a diagnostic.

30 The greatest difficulty with applying existing nucleic acid probe technology, and the reason that the utility of the existing probe technology is limited, is the copy number problem. In a virus or cell, for example, there is usually a single copy of a particular gene. This one copy may give rise to many copies of 35 gene product, either RNA or protein. For this reason, diagnostic techniques have often involved probing the protein, since the specific sequence of nucleic acid to be detected may give rise to many thousand copies of protein.

35 The naturally-occurring high number of ribosomal RNA, up to 100,000 copies per cell, has been used by GenProbe to facilitate diagnosis of certain bacterial pathogens, such as Legionella and Mycoplasma, using nucleic acid probes. However, this strategy cannot be used with non-cellular pathogens, such as viruses. Copy number is a particular problem with the development of a nucleic acid probe method for the detection of AIDS virus, where the integrated provirus may be present in less than one of ten thousand peripheral blood lymphocytes. Thus, if the particular nucleic acid sequence suspected to be present in a 40 sample could be amplified, the copy number problem could be circumvented and probe assays could be more readily used.

In a normal biological sample, containing only a few cells, and consequently only a few copies of a particular gene, it is necessary to utilize an amplification process in order to overcome the copy number problem.

45 One method to amplify is to 'grow out' the sample, that is, to arrange conditions so that the living biological material present in the sample can replicate itself. Replication increases the quantity of nucleic acid sequences to detectable levels. In the food industry, for example, in order to test processed food for the food-poisoning bacteria *Salmonella*, food samples must be incubated for a number of days to increase the quantity of nucleic acids. In clinical samples, pathogens must also be allowed to increase their number 50 by growing out over some considerable time.

United States Patent No. 4,683,195 issued on July 28, 1987 to Cetus Corporation and United States Patent No. 4,683,202 issued on July 28, 1987 to Cetus Corporation are each directed to a process for amplifying a target nucleic acid sequence contained in a sample. United States Patent No. 4,683,195 relates to a process in which a sample suspected of containing a target nucleic acid sequence is treated with 55 oligonucleotide primers such that a primer extension product is synthesized which in turn serves as a template, resulting in amplification of the target nucleic acid sequence. The primer extension product is separated from the template in the preferred embodiment using heat denaturation. Similarly, United States Patent No. 4,683,202 relates to a process for amplifying a target nucleic acid sequence having two separate

complementary strands. The process includes treating the strands with primers to synthesize extension products, separating the primer extension products from the templates, and in turn using the primer extension products as templates.

Both of the above United States patents require either manual or mechanical participation and multi-step operations by the user in the amplification process. The steps involved in these patents require the user to heat the sample, cool the sample, add appropriate enzymes and then repeat the steps. The temperature changes cause the enzymes to lose their activity. Hence, the user is required to repeatedly supplement the amplification mixture with aliquots of appropriate enzymes during the amplification process.

In addition, in United States Patents Nos. 4,683,195 and 4,683,202 each cycle of the amplification process takes place by the synthesis from a first template, of a second template, the second template in turn is used to synthesize the first template. This procedure is repeated, thus, each cycle of the amplification process is based on the synthesis of one product from one substrate.

Notwithstanding the amplification processes disclosed in the prior art, a need exists for improvements to the amplification process. It would be preferable if the amplification process required less participation and fewer manipulations by the user. Further, it would be advantageous if the amplification took place at a relatively constant ambient temperature so that the activity of the enzymes involved in the process would not be affected. It would be more expedient if a template could be used to generate more than one product from one substrate in each cycle of the amplification process.

Science 239 (1988) 491 describes a method of amplification of a nucleic acid sequence by means of the incorporation by PCR of a promoter sequence of a DNA-dependent RNA polymerase into a DNA copy of the sequence of interest, followed by the transcription of RNA copies. Reverse transcriptase copying of the RNA is envisaged as a method of sequencing the nucleic acid of interest.

SUMMARY OF THE INVENTION

This invention relates to an amplification process which is expedient and requires less participation and fewer manipulations by the user of the process than conventional amplification processes. The amplification takes place at a relatively constant ambient temperature. In addition, each cycle of the process generates a plurality of copies of product from one substrate. The amplification process of this invention may be used to increase the quantity of a specific nucleic acid thus circumventing the copy number problem. Hence, probe assays may be more readily used. The amplification process could also be used to increase the purity of a specific nucleic acid sequence as a substitute for conventional cloning methodology.

According to one aspect of the invention, a process for amplifying a specific nucleic acid sequence is used. The process involves the synthesis of single-stranded RNA, single-stranded DNA, and double stranded DNA. The single stranded RNA is a first template for a first primer. The single stranded DNA is a second template for a second primer. The double stranded DNA is a third template for the synthesis of a plurality of copies of the first template. A sequence of the first or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence and a sequence of the first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence. A 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

According to another aspect of the invention, a process for amplifying a specific nucleic acid sequence is used. The process involves:

- (a) hybridizing a first primer to a first template. The first primer has a DNA sequence which is sufficiently complementary to a RNA sequence of the first template;
- (b) synthesizing a first DNA sequence covalently attached to the first primer and complementary to the RNA sequence of the first template. The first DNA sequence and the first primer comprise a second template;
- (c) separating the first template from the second template to allow hybridization of a second primer;
- (d) hybridizing the second primer to the second template. The second primer has a DNA sequence which is sufficiently complementary to a DNA sequence of the second template. The second primer also has an antisense sequence of a promoter and an antisense sequence of a transcription initiation site for a RNA polymerase;
- (e) synthesizing a second DNA sequence covalently attached to the second primer and complementary to the DNA sequence of the second template and synthesizing a third DNA sequence covalently attached to the second template and complementary to the DNA sequence of the second primer. The second and third DNA sequences, the second primer and the second template comprise a third template;
- (f) synthesizing a plurality of copies of the RNA sequence of the first template from the third template.

A sequence of the first or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence and a sequence of the first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence. A 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

5 In a further alternative of the invention, the second primer of DNA has a sequence at its 3' end which is sufficiently complementary to the DNA sequence of the second template. The second primer has at its 5' end an antisense sequence of a promoter and an antisense sequence of a transcription initiation site for a RNA polymerase.

10 In a further alternative of the invention, the third DNA sequence covalently attached to the second template is complementary to the DNA sequence at the 5' end of the second primer.

15 In another alternative of the invention, a process for amplifying a specific nucleic acid sequence is used. The process involves combining a first primer, a second primer, ribonuclease H, a RNA-directed DNA polymerase, a DNA-directed DNA polymerase, a RNA polymerase, ribonucleoside triphosphates and deoxyribonucleoside triphosphates with a sample. The first primer of DNA has a sequence which is sufficiently complementary to a first template of RNA. The second primer of DNA has a sequence which is sufficiently complementary to a second template of DNA, and an antisense sequence of a promoter and an antisense sequence of a transcription initiation site which are recognized as substrate by the RNA polymerase. A sequence of the first primer or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence and a sequence of the first primer or the second primer is sufficiently homologous to a sequence of the specific nucleic acid. A 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

20 In a further alternative of the invention, a process for amplifying a specific nucleic acid sequence is used. The process involves adding a first primer, a second primer, avian myoblastosis viral polymerase, E. coli ribonuclease H, bacteriophage T7 RNA polymerase, ribonucleoside triphosphates and deoxyribonucleoside triphosphates to a sample. The first primer of DNA has a sequence which is sufficiently complementary to a first template of RNA. The second primer of DNA has a sequence which is sufficiently complementary to a second template of DNA, and an antisense sequence of a promoter and an antisense sequence of a transcription initiation site which are recognized as substrate by T7 RNA polymerase. A sequence of the first primer or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence and a sequence of the first primer or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence. A 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

BRIEF DESCRIPTION OF THE DRAWINGS

35 In drawings which illustrate embodiments of the invention,
Figure 1 is a general illustration the nucleic acid amplification process;
Figure 2 shows the synthetic oligonucleotides DNA sequences which are used for testing the amplification process: Figure 2A, the gag test sequence; Figure 2B, the gag2 test sequence;
40 Figure 3 is an autoradiogram of PAGE analysis of amplification reactions using different primer concentrations;
Figure 4 is an autoradiogram of PAGE analysis of amplification reactions using different template concentrations;
Figure 5 is an autoradiogram of Dot-blot hybridization on amplification reactions;
45 Figure 6 is an autoradiogram of PAGE analysis of amplification reaction using restriction fragments as template.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

50 This invention relates to a process for amplifying a specific nucleic acid sequence. The amplification involves the alternate synthesis of DNA and RNA and is generally illustrated in Figure 1. In this process, single-stranded RNA is converted to single-stranded DNA which in turn is converted to a functional template for the synthesis of a plurality of copies of the original single-stranded RNA. A first primer and a second primer are used in the amplification process. A sequence of the first primer or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence and a sequence of the first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence. In some instances, both the first primer and second primer are sufficiently complementary and sufficiently homologous to a sequence of the specific nucleic acid sequence, for example, if the specific nucleic acid

sequence is double stranded DNA.

The RNA is converted to single-stranded DNA by hybridizing an oligonucleotide primer (the first primer) to the RNA (the first template) and synthesizing a complementary strand of DNA from the first primer, (the first DNA sequence) by using a RNA-directed DNA polymerase. The resulting single-stranded DNA (the second template) is separated from the first template by, for example, hydrolysis of the first template and by using a ribonuclease which is specific for RNA-DNA hybrids (for example, ribonuclease H). The second template is converted to a form which is capable of RNA synthesis by hybridizing a synthetic oligonucleotide (the second primer), which contains at its 3' end a sequence which is sufficiently complementary to the 3' end of the second template and toward its 5' end a sequence containing the antisense strand of a promoter and antisense sequence of a transcription initiation site, and by synthesizing a second DNA sequence covalently attached to the 3' end of the second primer using the second template as a template and synthesizing a third DNA sequence covalently attached to the 3' end of the second template using the second primer as a template, using DNA-directed DNA polymerase. The resulting functional derivative of the second template, which is a third template, is used for the synthesis of a plurality of copies of RNA, the first template, by using a RNA polymerase which is specific for the promoter and transcription initiation site defined by the second primer. Each newly synthesized first template can be converted to further copies of the second template and the third template by repeating the cycle. In addition, repetition of the cycle does not require participation or manipulation by the user.

The amplification process commences with the addition of a suitable template nucleic acid to the appropriate enzymes, primers, and cofactors under the appropriate reaction conditions. This template nucleic acid is in a form which is capable of homogenous and continuous amplification and can function as an intermediate in the cycle set forth in Figure 1. The amplification process involves the net consumption of precursors (primers, ribonucleoside triphosphates and deoxyribonucleoside triphosphates) and the net accumulation of products (RNA and DNA). The processes of RNA and DNA synthesis will proceed asynchronously until sufficient levels of nucleic acids have been synthesized to allow detection. The amplification process may be monitored by, for example, the synthesis of a labeled product from a labeled precursor.

It is contemplated that amplification may involve another process either in addition to or in place of the one generally illustrated in Figure 1. Also possible are certain counter-productive enzymatic reactions which occur at permissibly low rates. Included among the possible non-productive side reactions is the synthesis of RNA and/or DNA in the absence of an added template nucleic acid. Such RNA and/or DNA products can be discriminated from desired products by determining whether a particular sequence which would be found only between the two priming sites of the specific nucleic acid sequence is present.

The first primer is an oligodeoxyribonucleotide which has at its 3' end a sequence which is sufficiently complementary to the 3' end of the first template. The sequence at the 3' end of the first primer has a particular length and base composition to allow specific and efficient synthesis of the first DNA sequence, under the given conditions of ionic strength and temperature. The first primer may be sufficiently complementary to a region internal to the 3' end of the first template in the first cycle. In subsequent cycles, the 5' end of the first primer would be complementary to the 3' end of the first template. It is contemplated that the first primer may be composed partially or completely of nucleotides or nucleotide analogs other than the natural deoxyribonucleotides. The 5' end of the first primer may contain sequences which are not complementary to the first template in the first cycle. The non-complementary sequences may be complementary to a nucleic acid which can be immobilized, or to which can be bound a useful non-nucleic acid component, such as a reporter to facilitate detection. Alternatively, the non-complementary sequences may include an antisense sequence of a promoter and an antisense sequence of a transcription initiation site, which could be used for the synthesis of RNA. This RNA would be complementary to the first template and could be used as an intermediate in another amplification cycle.

The second primer is an oligodeoxyribonucleotide which contains at its 3' end a sequence which is sufficiently complementary to the 3' end of the second template. The second primer has a particular length and base composition to allow specific and efficient synthesis of the second and third DNA sequences, under the given conditions of ionic strength and temperature. In addition, the second primer contains the antisense sequence of a functional promoter and the antisense sequence of a transcription initiation site. This sequence, when used as a template for synthesis of the third DNA sequence, contains sufficient information to allow specific and efficient binding of a RNA polymerase and initiation of transcription at the desired site. The promoter sequence may be derived from the antisense strand of a functional promoter. The transcription initiation site may be derived from the 5' terminal sequence of a natural RNA transcript. In the preferred embodiment, the 5'-terminal sequence of the second primer is AATTCTAATACGACTCAC-TATAGGGAG. This sequence contains the antisense sequence of the promoter and the antisense sequence

of the transcription initiation site for T7 RNA polymerase. Alternatively, the transcription initiation site and promoter for another phage RNA polymerase may be used. In addition, sequences which are unrelated to the promoter function may be included at the 5' end of the second primer or between the transcription initiation site and the sequence at the 3' end which hybridizes to the second template. It is contemplated
 5 that the second primer may be composed partially or completely of nucleotides or nucleotide analogs other than natural deoxyribonucleotides.

All of the enzymes used in this invention should meet certain practical specifications. Each enzyme or enzyme preparation should be free of deleterious deoxyribonuclease ("DNase") activities, such as the 5' or 10 3' exonuclease activities which are often associated with certain DNA polymerases and single-strand or double-strand specific exonuclease or endonucleases. Each enzyme or enzyme preparation should be free of deleterious ribonuclease ("RNase") activities, with the exception of the preferred addition of a ribonuclease activity which is specific for hybrids of RNA and DNA (for example, ribonuclease H). In addition, each enzyme should be reasonably active under the common reaction conditions which are used for the other enzymatic processes, and non-enzymatic processes, such as hybridizing oligonucleotide
 15 primers to the RNA or DNA templates.

The DNA-directed RNA polymerase which is used in this invention may be any enzyme capable of binding to a particular DNA sequence called a promoter and specifically initiating *in vitro* RNA synthesis at a defined initiation site within close proximity to the promoter. The promoter and the initiation site form part of the second primer. In addition the RNA polymerase should be capable of synthesizing several copies of
 20 RNA per functional copy of template in a reasonable amount of time. In the preferred embodiment, the bacteriophage T7 RNA polymerase is used. In addition other bacteriophage RNA polymerases, such as phage T3, phage ϕ II, Salmonella phage sp6, or Pseudomonas phage gh-1 may be used. In another embodiment, other prokaryotic or eukaryotic DNA-directed RNA polymerase may be used. It should be understood that if alternative RNA polymerases are used, then the necessary changes to the promoter and
 25 initiation sequences of the second primer should be made according to the template specificity of the particular RNA polymerase.

The RNA-directed DNA polymerase which is used in this invention may be any enzyme capable of synthesizing DNA from an oligodeoxyribonucleotide primer and a RNA template. In addition this enzyme may contain activities for DNA-directed DNA polymerase and RNase H. In the preferred embodiment, the
 30 avian myoblastosis viral polymerase ("AMV reverse transcriptase") is used. In addition, the RNA-directed DNA polymerase could be from another retrovirus, such as Maloney murine leukemia virus. Alternatively, other eukaryotic RNA-directed DNA polymerases could be used.

The DNA-directed DNA polymerase which is used in this invention may be any enzyme capable of synthesizing DNA from an oligodeoxyribonucleotide primer and a DNA template. This enzyme should not
 35 contain either 5'- or 3'- exonuclease activities, which are associated with many types of DNA polymerase. In the preferred embodiment, the AMV reverse transcriptase is used. However, other DNA-directed DNA polymerases which naturally lack the 5'- or 3'- exonuclease activities could be used. These could include certain eukaryotic DNA polymerases, such as, DNA polymerase γ or β those DNA polymerases which could be isolated from a mammalian tissue, such as calf thymus. An otherwise unsuitable DNA polymerase
 40 could be made useful by removing the undesirable exonuclease activities either by alteration of the DNA polymerase gene followed by expression of the altered polymerase in a suitable host cell, or by chemical modification of the DNA polymerase protein. Altered versions of DNA polymerase could be made from the Klenow fragment of *E. coli* DNA polymerase I or the bacteriophage T7 DNA polymerase. It should be understood that such alternative DNA-directed DNA polymerase activities are added to supplement the
 45 activity contributed by the RNA-directed DNA polymerase, since in the preferred embodiment, both RNA-directed and DNA-directed DNA polymerase activities are supplied by the same enzyme.

The RNase H which could be used in this invention may be any enzyme capable of hydrolyzing a RNA which is annealed to a complementary DNA. This enzyme should not be capable of hydrolyzing single or double-stranded RNA or any DNA. In the preferred embodiment, the *E. coli* RNase H is used. In addition, other RNase H enzymes could be used, such as calf thymus RNase H. Since RNase H is an intrinsic activity of AMV reverse transcriptase, the *E. coli* RNase H will be supplemented in the preferred embodiment by the RNase H of AMV reverse transcriptase. Alternatively, any other enzyme capable of separating the second template from the first template could be used.

The abovementioned enzymes and primers are mixed together in a reaction vessel which contains the
 55 necessary buffers and cofactors for both DNA and RNA synthesis. In addition, the ionic conditions and reaction temperature should be compatible with specific hybridization of the primers to the DNA and RNA templates as is known to those skilled in the art. The reaction mixture should be free of such agents which would interfere with the amplification process, specifically substances which could greatly inhibit the activity

of the enzymes, interfere with the hybridizing of primers and templates, or degrade non-productively the nucleic acid intermediates and products.

5 The description of possible detection schemes may be useful to the application of the amplification process. It should be understood that schemes which may be used for detecting the nucleic acids which are synthesized in the amplification process are not limited to those described herein, and it is contemplated that other methods may be used.

10 In one embodiment, a labeled precursor may be added to the reaction mixture. Amplification is determined by quantitative or qualitative analysis of labeled products, which can be separated from the labeled precursor by using methods known in the art. A labeled precursor may be a ribonucleoside triphosphate for detecting RNA synthesis, or a deoxynucleoside triphosphate or an oligonucleotide primer for detecting DNA synthesis. The type of label may be a radioisotope or a useful chemical group, such as biotin, a chromophore, a fluorophore, or a hapten which could bind to an antibody, or possibly a protein or an enzyme. The labeled products may be separated from the labeled precursors on the basis of solubility, 15 charge, or size. In addition, the labeled DNA or RNA may be hybridized to a nucleic acid which contains a complementary sequence and which can be immobilized.

20 In another embodiment, the products of the amplification process may be bound to an immobilized support, hybridized to a nucleic acid probe containing a complementary sequence, and separated from the unhybridized nucleic acid probe which remains in solution. The products, DNA or RNA, may be bound directly to a solid support by any stable interaction, such as hydrophobic, electrostatic, or covalent 25 interaction. In addition, the products may contain certain chemical groups, for example, biotin, which may be incorporated into the products during the amplification process to allow binding to an immobilized protein, for example, avidin or streptavidin. In addition, the products may be hybridized to a nucleic acid which contains a complementary sequence and which can be immobilized. The nucleic acid probe would contain a complementary sequence which forms a sufficiently stable interaction with a product of the 30 amplification process to allow binding under the conditions of hybridization and sustained binding under the conditions used for removal of the unhybridized nucleic acid probe. In the preferred embodiment the complementary sequence would be derived from that part of the specific nucleic acid sequence which is between the sequences of the first primer and the second primer. The nucleic acid probe may be a single-stranded DNA or RNA, or a double-stranded DNA or RNA which can be made single-stranded, or an 35 oligonucleotide which can be composed of deoxyribonucleotides and/or ribonucleotides. In addition, the nucleic acid probe may contain a chemical group which could covalently bind to a product DNA or RNA under the appropriate conditions. The nucleic acid probe may be labeled with a radioisotope or a useful chemical group, such as biotin, a chromophore, a fluorophore, or a hapten which could bind to an antibody. In addition, the nucleic acid probe could be conjugated to a protein or enzyme, for example, a phosphatase or a peroxidase. In addition, the nucleic acid probe may contain sequences which would allow in vitro replication of the probe.

40 It is contemplated that the products of the amplification process may be analyzed by methods which are typically used for nucleic acids that have been enriched by molecular cloning techniques. In one alternative, the synthesis of a specific DNA sequence may be detected by digestion of the synthesized DNA with a restriction endonuclease, followed by electrophoretic separation and detection using methods known in the art. In another alternative, the sequence of amplified RNA may be determined by DNA synthesis using a RNA-directed DNA polymerase, the first primer, and dideoxynucleoside triphosphates (Stoflet et al., 1988). In another alternative, the sequence of the amplified third template may be determined by RNA synthesis using the DNA-directed RNA polymerase used in the amplification process, and 3'-45 deoxyribonucleoside triphosphates (Axelrod & Kramer, 1985). In another alternative, the amplified RNA may encode a polypeptide which could be translated, in vitro. The polypeptide product of the in vitro translation could be analyzed by using an antibody.

50 A sample suspected of containing or known to contain the specific nucleic acid sequence is added to the reaction mixture in the form of a template nucleic acid which is capable of homogeneous and continuous amplification and may be any intermediate in the cycle set forth in Figure 1. In particular, the template nucleic acid may be a single-stranded RNA which contains at its 5' end a sequence which is sufficiently homologous to that which is at the 3' end of the second primer, and contains a sequence which is sufficiently complementary to the first primer. A template nucleic acid of this form would function as a first template in the amplification process. Alternatively, the template nucleic acid may be a single-stranded 55 DNA which contains at its 3' end a sequence which is sufficiently complementary to at least the 3' end of the second primer, and contains a sequence which is sufficiently homologous to that which is at the 3' end of the first primer. A template nucleic acid of this form would function as a second template in the amplification process. Alternatively, the template nucleic acid may be a double-stranded DNA, one strand of

which contains at its 5' end the entire sequence of the second primer and contains a sequence which is sufficiently complementary to the first primer. The double-stranded DNA functions as a third template in the amplification process.

Although the preparation of a template nucleic acid is not part of the amplification process, the description of possible schemes for generating template nucleic acids may be useful to the application of the amplification process. It should be understood that the schemes which may be used for obtaining the template nucleic acid are not limited to the alternatives which are described herein, and it is contemplated that other methods may be used.

In one alternative, a template nucleic acid which could function as a first template could be a naturally occurring RNA or a RNA fragment which could be generated from a larger RNA molecule by using site specific hydrolysis methods known in the art (Shibahara et al., 1987).

In another alternative, a template nucleic acid which could function as a second template could be generated from a double-stranded DNA by digestion with a restriction endonuclease which has a site immediately flanking the sequence which is sufficiently complementary to the 3' end of the second primer.

The resulting double-stranded DNA fragments could then be made single-stranded by using chemical or thermal denaturation methods.

In another alternative, a template nucleic acid which could function as a second template could be generated from a single-stranded DNA or RNA to which has been hybridized an oligonucleotide which is capable of blocking DNA synthesis. This blocking oligonucleotide may contain a chemical group, which could covalently bind to the template, under the appropriate conditions. DNA synthesis from this blocked template using the first primer could result in a synthesized DNA with the same 3' end as the second template. If the original template is RNA, then the resulting DNA-RNA hybrid may be used directly as a template nucleic acid. If the original template is DNA, then the resulting copy of the second template could then be separated from the original template by using chemical or thermal denaturation methods.

In another alternative, a template nucleic acid which could function as a third template could be generated from a single-stranded DNA or RNA by DNA synthesis from the DNA or RNA template using the second primer. The resulting synthesized DNA could then be separated from the original template by using chemical or thermal denaturation methods. In addition, a RNA template could be hydrolyzed by using chemical or enzymatic methods. The resulting single-stranded DNA has the sequence of the second primer covalently attached to its 5' end and contains a sequence which is sufficiently complementary to the first primer. This single-stranded DNA could be converted to a transcriptionally functional double-stranded DNA by hybridizing the first primer to the single-stranded DNA, and by synthesizing a DNA sequence which is covalently attached to the first primer and complementary to the single-stranded DNA.

In a further alternative, a single-stranded DNA or RNA template could be obtained from a double-stranded DNA, double-stranded RNA or a DNA-RNA hybrid by using chemical, thermal, or possibly enzymatic methods. Then, by using one of the alternative schemes proposed above, the resulting single-stranded DNA or RNA could then be used to generate a template nucleic acid which could function as a first, second or third template. In addition, an alternative scheme involving the first primer and one strand of nucleic acid, and another alternative scheme involving the second primer and the other (complementary) strand of the nucleic acid may be used concurrently to generate template nucleic acids.

MATERIALS AND METHODS

Materials

Oligonucleotides were synthesized using an Applied Biosystems 380A DNA synthesizer. Columns, phosphoramidites, and reagents used for oligonucleotide synthesis were obtained from Applied Biosystems, Inc. through Technical Marketing Associates. Oligonucleotides were purified by polyacrylamide gel electrophoresis followed by DEAE cellulose chromatography. The radioisotope [α -32p] UTP (800 Ci/mmol) was from Amersham. Enzymes for digesting and ligating DNA were purchased from New England Biolabs, and used according to the supplier's recommendations. Preparations containing the large fragment of DNA polymerase 1 (Klenow) were also purchased from New England Biolabs. RNasin and T7 RNA polymerase from Promega Biotec were purchased through Bio/Can Scientific Inc. Reverse transcriptase and RNase H were obtained from Pharmacia. The supplier for proteinase K was Boehringer Mannheim Canada. E. coli strain HB101 (ATCC 33694) was used for all transformations. The plasmid pUC19 (Norrand et al., 1983) was purchased from Bethesda Research Laboratories.

Isolation of DNA and sequencing

5 E. coli transformants were grown on YT medium (Miller, 1972) containing 50 ug/ml ampicillin. Plasmid DNA was purified by a rapid boiling method (Holmes and Quigley, 1981). DNA fragments and vectors used for all constructions were separated by electrophoresis on low melting point agarose, and purified from the molten agarose by phenol extraction and ethanol precipitation (Maniatis et al., 1982). Plasmid DNA was sequenced using a modification (Hattori et al., 1985) of the dideoxy method (Sanger et al., 1977). Reactions were run using the -20 universal primer (New England Biolabs).

10 **TCA precipitation**

15 Aliquots (5ul) of amplification reactions were quenched in 20ul 10 mM EDTA and placed on ice until all time point samples had been collected. The quenched samples were then applied to glass filter discs, and immediately dropped into ice-cold 5% trichloroacetic acid ("TCA") - 1% sodium pyrophosphate for 10 min with occasional mixing. Two 5 min washes with ice-cold 5% TCA were followed by two additional washes with 95% ethanol and lyophilization to dryness. Radioactivity was determined in a liquid scintillation counter.

Polyacrylamide gel electrophoresis

20 Samples (1 to 6 ul) were mixed with 4-5 ul formamide dye (90% deionized formamide, 10 mM TrisHCl (pH 8.0), 1 mM EDTA, xylene cyanol and bromophenol blue), and applied to a pre-run 12-cm- long 7% denaturing polyacrylamide gel. Gels were run at 350 volts until the bromophenol blue dye had reached the bottom. In some cases the gels were fixed and dried prior to autoradiography. Fixing involved a 15 min wash in 10% methanol- 7% acetic acid. The profiles of the RNA products separated by this procedure were 25 visualized by autoradiography at room temperature.

Example 1: Design and synthesis of oligonucleotides for a gag test system

30 A synthetic DNA sequence (Figure 2A) was designed to include an EcoRI site, a T7 phage promoter, a sequence required for initiation of transcription by T7 RNA polymerase and a 19 bp hybridization region (hybridization region 1). The 47 b antisense strand oligonucleotide (T7H1.GAG) involved in the cloning of these elements also serves as the first primer. Hybridization region 2 lies 53 bp away from hybridization region 1 and is 20 bp in length. The primer made to this region (H2.GAG) is a 20 b oligonucleotide duplicate of the sense strand and is not used for cloning. The sequence spanning and including the 35 hybridization regions is a 92 bp segment of the gag portion of the HTLV-III genome, the causative agent of AIDS. This particular gene segment was chosen because the primers were predicted to hybridize efficiently and because the distance between the two hybridization regions was relatively short. In addition, a XbaI site was placed at the end of the sequence for cloning ease. The gag test sequence also contains SphI and PstI sites which can aid in the screening of recombinants.

40 A total of four oligonucleotides were used in the cloning of this fragment. N1.GAG, used in the construction of both the gag test and the gag2 test sequence, completes the antisense strand and is used only in the cloning process. Similarly, T74.PRO is the sense strand component of the T7 promoter. N2.GAG however, was used in the construction of both test fragments, and has also been used as an intermediate (second template) in two steps of the amplification cycle. The entire cloned gag test fragment can also 45 represent an intermediate of the amplification cycle (third template). Once cloned into an appropriate vector the gag test DNA could be transcribed by T7 RNA polymerase to produce a RNA fragment (first template) useful as an amplification intermediate involved in three of the steps. In addition, T7H1.GAG and H2.GAG serve as primers in the test system.

50 The gag2 test synthetic DNA fragment (Figure 2B) does not contain the T7 promoter, but the remainder of the sequence is identical to the gag test sequence and therefore, both N1.GAG and N2.GAG were involved in its construction. The oligonucleotide required to complete the antisense strand is called H1.GAG. Once cloned, the gag2 test fragment can be used as a template for testing amplification, using a DNA restriction fragment as template nucleic acid.

55 Example 2: Construction of the gag test plasmids

The oligonucleotides T74.PRO and N1.GAG (2 ug each), were phosphorylated separately in 20 ul reactions containing 70 mM TrisHCl (pH 7.6), 10 mM MgCl₂, 5 mM DTT, 0.5 mM ATP and 5 units T4

polynucleotide kinase, at 37°C for 30 min. Phosphorylated T74.PRO and N1.GAG (10 μ l of each) were mixed with 1 μ g each of unphosphorylated T7H1.GAG and N2.GAG, and 3 μ l 100 mM TrisHCl (pH 7.8) - 500 mM NaCl, in a final volume of 29 μ l for the gag test assembly. The gag2 test mixture contained 10 μ l phosphorylated N1.GAG, 1 μ g each of unphosphorylated H1.GAG and N2.GAG, and 1.8 μ l 100 mM TrisHCl (pH 7.8) - 500 mM NaCl, in a final volume of 18 μ l. The oligonucleotide mixtures were hybridized separately by placing them at 90°C for 10 min followed by slow cooling to room temperature for 10 to 16 h 60 μ l reactions containing 50 mM TrisHCl (pH 7.8), 10 mM MgCl₂, 20 mM DTT, 1mM ATP and 50 μ g/ml BSA were used to ligate the hybridized oligonucleotides together. 400 units T4 DNA ligase was added to the gag test reaction and it was incubated at 15°C for 2h while the gag2 test reaction was incubated for 14 to 16 h with 200 units T4 DNA ligase.

5 The isolated and purified synthetic DNA segments were mixed with plasmid pUC19 which had been linearized by digestion at restriction enzyme sites within the polylinker region. T4 DNA ligase was used to ligate the gag test sequence into the EcoRI- XbaI fragment of pUC19, while the gag2 test sequence was 10 ligated to the SmaI-XbaI fragment. Plasmid DNA from transformants obtained after these reactions were 15 used to transform E. coli were screened by restriction analysis , and the final plasmids (pGAG.TEST and pGAG2.TEST) were determined to be correct by sequence analysis.

Example 3: Effect of primer concentration on RNA amplification

20 The reaction mixtures (25 μ l) which were used to amplify RNA transcribed from the gag test oligonucleotides contained 50 mM TrisHCl (pH 8.45), 6 mM MgCl₂, 40 mM KCl, 10 mM dithiothreitol, 0.5 mM NTP (ATP, CTP, GTP, UTP), 1 mM dNTP (dATP, dCTP, dGTP dTTP), 20 units RNasin, 10 units T7 RNA polymerase, 10 units reverse transcriptase, 0.4 units RNase H, and 10 μ Ci [α -32p] UTP. Two of the reactions contained 0.5 ng (0.015 pmoles) N2.GAG while the other two reactions contained no template. 25 The primers T7H1.GAG and H2.GAG were each added at final concentrations of 3.4 μ M or 0.34 μ M to reactions containing either N2.GAG or no template. The reactions were incubated at 42°C for 2 h. Total synthesis of RNA was monitored by determining the incorporation of TCA insoluble cpm at 30 min. intervals. The effect of the primer concentration on template- dependent RNA synthesis is shown in Table 1. Aliquots of each reaction, containing equal amounts of synthesized RNA, were analyzed by PAGE and 30 autoradiography (Figure 3, lanes 1-4 numbered the same as the reactions).

Table 1

RNA amplification from N2.GAG after 2 h.			
Reaction	Concentration of each primer (μ M)	Template (ng)	RNA Synthesized (ug)
1	3.4	0.5	2.8
2	3.4	-	2.1
3	0.34	0.5	1.8
4	0.34	-	0.7

35 It was found that while reaction 1 resulted in the greatest incorporation of isotope, the no template control, reaction 2, was also high (73% of reaction 1) and produced a very similar electrophoretic profile. It 40 would therefore appear that in the presence of high primer concentrations, a RNA transcript of identical size to that expected in amplification is produced in the absence of any template. Results using samples with a 10- fold decrease in primer concentration were dramatically different. The amount of RNA produced in reaction 3 was 2.6 times that of reaction 4, but while virtually all of the transcript was found in a single band of the expected size in reaction 3, no fragments greater than 60 to 70 b were found in reaction 4. Primer 45 concentration therefore plays a significant role in the accuracy and efficiency of RNA amplification.

50 A control RNA transcript used to represent the size of fragment expected to be generated by the amplification system (lane 0 of Figure 3) was prepared by transcription from the test plasmid. pGAG.TEST was linearized by digestion with XbaI, proteinase K treated (Maniatis et al., 1982), phenol extracted and ethanol precipitated. T7 RNA polymerase was then used according to the supplier's recommendations to 55 transcribe 0.5 ug of the resulting fragment in a 25 μ l reaction mixture containing 10 μ Ci [α -32p] UTP.

Example 4: Effect of template concentration on RNA amplification

5 The standard 50 μ l reaction mixture used to amplify RNA transcribed from the gag test oligonucleotides contained 0.34 μ M T7H1.GAG, 0.34 μ M H2.GAG, 50 mM TrisHCl (pH 8.45), 6 mM MgCl₂, 40 mM KCl, 10 mM DTT, 0.5 mM NTP, 1 mM dNTP, 40 units RNasin, 20 units T7 RNA polymerase, 20 units reverse transcriptase, 0.8 units RNase H and 10 - 20 μ Ci [α -32p] UTP. The reactions contained amounts of template (N2.GAG) varying from 1 ng to 1 fg. One reaction contained no template. The reactions were incubated at 42°C for 3h, during which total synthesis of RNA was monitored by determining the incorporation of TCA insoluble cpm at 30 min intervals. As indicated in Table 2, total RNA synthesis was higher than the no template control for all template concentrations tested. Although the total synthesis of RNA generally decreased with decreasing template concentration, this decrease in synthesis was not quantitative. Thus, the degree of amplification of RNA per starting template generally increased with decreasing template concentration. Amplification of 8×10^8 fold was achieved by synthesis of 0.8 μ g RNA from 1 fg of N2.GAG template. One fg of the 102-b N2.GAG oligonucleotide represents approximately 2×10^4 molecules.

15

Table 2

RNA amplification from N2.GAG after 3h.				
	Reaction	Template	RNA Synthesized (ug)	Fold amplification
20	1	1 ng	3.5	3.5×10^3
	2	100 pg	4.4	4.4×10^4
	3	10 pg	4.1	4.1×10^5
	4	1 pg	3.0	3.0×10^6
	5	100 fg	2.7	2.7×10^7
	6	10 fg	1.9	1.9×10^8
	7	1 fg	0.78	7.8×10^8
	8	-	0.046	-

30

30 The RNA which was synthesized after a reaction time of 3h was analyzed by PAGE, for each template concentration (Figure 4 lanes 1-8, numbered the same as the reactions). A major band representing a RNA of about 100 b was present in all reactions except the ones containing 1 fg template and no template. The reaction containing 1 fg template did not have much of this 100 b product at 3 h but the overall RNA synthesis was higher than and qualitatively different from the no template reaction.

Example 5: Hybridization analysis of RNA products.

40 Amplification reactions containing amounts of N2.GAG template varying from 1 pg to 0.1 fg were performed following the teaching of example 4, except the radiolabeled UTP was omitted. The reactions were incubated at 42°C for 3 h. Aliquots were removed from each reaction at 30 min intervals and applied to a nylon membrane (Amersham). The nucleic acids that were contained in these reaction aliquots were fixed by exposure to ultraviolet light. The membrane was prehybridized at 50°C for 1 h in prehybridization buffer consisting of a final concentration of 50% v/v formamide, 5 X SSC and 5 X Denhardt's solution (Maniatis *et al.*, 1982; Southern *et al.*, 1975) at a volume equivalent to 5 mls of solution per 100 cm² and hybridized with a radiolabeled probe with a specific activity of 10^6 cpm/ml of hybridization solution. Hybridization was performed at 50°C for 16 h in 50% formamide, 5 X SSC and 5 X Denhardt's solution (Maniatis *et al.*, 1982; Southern *et al.*, 1975). The radiolabeled probe was the synthetic oligonucleotide 5' GATCTGGGATAGAGTACATCCA 3' which had been labeled at the 5' end using T4 polynucleotide kinase and (α -32p) ATP. After the membrane was washed at 50°C in a series of 2, 3 min. washes consisting of 2 X SSC, 0.1% v/v SDS and 0.2 X SSC, 0.1% v/v SDS (Southern *et al.*, 1975; Maniatis *et al.*, 1982; Szostak *et al.*, 1979).

45 Figure 5 shows the results of the hybridization analysis performed on the amplification reactions, containing various amounts of N2.GAG template, which were sampled at different times of incubation.

50 Each column of Figure 5 represents a different time point (1, 30 min; 2, 60 min; 3, 90 min; 4, 120 min; 5, 150 min; 6, 180 min) and each row represents a different amount of added N2.GAG template (1, 1pg; 2, 100 fg; 3, 10 fg; 4, 1 fg; 5, 0.1 fg; 6, no template). Amplification of nucleic acids which hybridized to the labeled probe were observed for rows 1-3 (1 pg - 10 fg), however the hybridization to specific nucleic acids

in rows 4 - 5 (1 fg, 0.1 fg) was not higher than row 6 (no template). The apparent non-specific binding of labeled probe in row 6 seems to be related to DNA or RNA synthesis since the hybridization signal increases with increasing time.

5 Example 6: Use of DNA restriction fragment as template.

The plasmid pGAG2.TEST was digested with Mspl, treated with proteinase K, purified by phenol extraction and ethanol precipitation, and denatured by boiling for 5 min. Amplification reactions were performed and analyzed following the teaching of Example 4, except the Mspl digested pGAG2.TEST was 10 used as a template instead of the N2.GAG oligonucleotide. The amounts of plasmid added to each reaction varied from 55 ng to 5.5 pg, and no template. To simulate additional DNA which would be present in an actual sample, alternate reactions contained 1 ng of calf thymus DNA which had been similarly digested, purified and denatured. After a 3 h incubation at 42°C, the synthesis of RNA was determined by TCA precipitation and PAGE analysis. As indicated in Table 3, total RNA synthesis was higher than the no 15 template controls for all template concentrations tested. The degree of amplification was calculated based on RNA synthesis from the actual template which was 1.8% of the total plasmid DNA.

The total RNA synthesis (degree of amplification) from a particular initial level template concentration was consistently lower for the restriction fragment (Table 3) as compared to that for the synthetic 20 oligonucleotide template (Table 2). This could be due to competition with the complementary strand of the restriction fragment template under the conditions used.

Table 3

25 RNA amplification from Mspl-digested pGAG2.TEST			
	Reaction	Template*	RNA Synthesized**
30	1	55.0 ng [1 ng]	3.65
	2		(4.05)
	3	5.5 ng [100 pg]	3.54
	4		(3.16)
	5	550.0 pg [10 pg]	2.29
	6		(2.79)
	7	55.0 pg [1 pg]	2.62
	8		(0.67)
	9	5.5 pg [100 pg]	1.37
	10		(2.26)
	11	-	1.25
	12		(0.08)

40 * Numbers in brackets indicate equivalent amounts of N2.GAG.

** Numbers in parentheses indicate RNA synthesis in presence of 1 ug
Mspl-digested calf thymus DNA.

45 The RNA which was synthesized after a reaction time of 3h was analyzed by PAGE (Figure 6, lanes 1-6, 11 and 12, numbered the same as the reactions). The major band representing a RNA of about 100 b was present in reactions (lanes) 1-6 but absent in the no template reactions (lanes 11 and 12). The RNA in lane 0 was a standard, which was prepared following the teaching of Example 3. There was no apparent qualitative difference in the synthesized RNA either with (lanes 2, 4 and 6) or without (lanes 1, 3, and 5) the additional of 1 ug of Mspl-digested calf thymus DNA.

50 Example 7: Use of RNA fragment as template

The plasmid pGAG.TEST is digested with XbaI, treated with proteinase K, and purified by phenol extraction and ethanol precipitation. RNA of a sequence complementary to N2.GAG is transcribed from the linearized pGAG.TEST plasmid using T7 RNA polymerase. The resulting RNA is purified by digestion with DNase (Pro Mega BioTec), followed by phenol extraction and ethanol precipitation. The purified RNA is used as a template for amplification reactions following the teaching of Example 5. Amounts of RNA are added to each reaction and vary from 55 ng to 5.5 pg, and no template. After a 3 h incubation at 42°C, the

synthesis of specific RNA is determined by hybridization to labeled oligonucleotide probe, following the teaching of Example 5.

Example 8: Use of ribosomal RNA as a template; amplification of internal sequences

5 Two primers are used for amplifying RNA sequences which are complementary to a part of *E. coli* 16S ribosomal RNA (rRNA). One of these primers T7HIRIB3.PR2 (AATTCTAATACGACTCACTATAGGGAGTATTACCGCGGCTGCTG) contains the antisense strand of the T7 promoter and initiation site, and a sequence which is complementary to 16S rRNA. The other RIB8.PR (AATACCTTGCTCATTGACG) is complementary to the DNA synthesized by using T7HIRIB3.PR2 as a primer and 16S rRNA as a template. A third synthetic oligonucleotide RIB5.PR (AGAACGCACCGGCTAAC) which allows detection of amplification is complementary to the RNA products of the amplification reaction, which are in turn complementary to the original rRNA template.

10 Reaction mixtures (25 μ l) contain 50 mM TrisHCl (pH 8.45), 6 mM MgCl₂, 40 mM KCl, 10 mM DTT, 0.5 mM NTP, 1 mM dNTP, 20 units RNasin, 10 units T7 RNA polymerase, 10 units AMV reverse transcriptase, 15 0.4 units RNase H, 0.34 μ m T7HIRIB3.PR2, and 0.34 μ m RIB8.PR.

15 Amounts of *E. coli* rRNA varying from 50 ng to 50 fg are added to the reactions. One reaction contains no added rRNA. The reactions are incubated at 42° for 3 h, during which aliquots are removed at 30, 60, 120, and 180 minutes. The reaction aliquots are quenched, fixed to a nylon membrane, and hybridized to 20 the ³²P 5'-end labeled RIB5.PR probe, following the teaching of Example 5.

Example 9: Use of ribosomal RNA as a template; amplification of 5'-terminal sequences.

25 Two primers are used for amplifying RNA sequences which are homologous to a part of *E. coli* 16S rRNA. One of these primers RIB12.PR (TTACTCACCGTCCGCC) is complementary to 16S rRNA. The other T7H1RIB5.PR (AATTCTAATACGACTCACTATAGGGAGAAATTGAAGAGTTGATCAT) is complementary to the 3'end of the DNA synthesized by using RIB12.PR as a primer and 16S rRNA as a template. A third synthetic oligonucleotide RIB11.PR (GTTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCC) which allows detection of amplification is complementary to both the RNA products of the amplification and the original rRNA template. The 30 amplification reactions for rRNA and detection of the synthesized RNA are performed following the teaching of Example 8, except that T7H1RIB5.PR and RIB12.PR are used as primers (in place of T7H1RIB3.PR2 and RIB8.PR) and RIB11.PR is used as an oligonucleotide probe (in place of RIB5.PR).

35 Although preferred embodiments of the invention have been described in detail, it will be understood by those skilled in the art that variations may be made thereto without departing from either the spirit of the invention or the scope of the appended claims.

Claims

40 1. A process for the amplification of a specific nucleic acid sequence, at a relatively constant temperature and without serial addition of reagents, comprising the steps of:

(A) providing a single reaction medium containing reagents comprising

(i) a first oligonucleotide primer,

(ii) a second oligonucleotide primer comprising an antisense sequence of a promoter recognized by an RNA polymerase,

(iii) a DNA-directed RNA polymerase that recognizes said promoter,

(iv) an RNA-directed DNA polymerase,

(v) a DNA-directed DNA polymerase,

(vi) a ribonuclease that hydrolyzes RNA of an RNA-DNA hybrid without hydrolyzing single- or double-stranded RNA or DNA, and

(vii) ribonucleoside and deoxyribonucleoside triphosphates;

then

(B) providing in said reaction medium RNA comprising an RNA first template which comprises said specific nucleic acid sequence or a sequence complementary to said specific nucleic acid sequence, under conditions such that a cycle ensues wherein

(i) said first oligonucleotide primer hybridizes to said RNA first template,

(ii) said RNA-directed DNA polymerase uses said RNA first template to synthesize a DNA second template by extension of said first oligonucleotide primer and thereby forms an RNA-DNA hybrid

intermediate,

(iii) said ribonuclease hydrolyzes RNA which comprises said RNA-DNA hybrid intermediate,

(iv) said second oligonucleotide primer hybridizes to said DNA second template,

(v) said DNA-directed DNA polymerase uses said second oligonucleotide primer as template to synthesize a functional promoter recognized by said RNA polymerase by extension of said DNA second template; and

(vi) said DNA-directed RNA polymerase recognizes said functional promoter and transcribes said DNA second template, thereby providing copies of said RNA first template;

and thereafter

(C) maintaining said conditions for a time sufficient to achieve a desired amplification of said specific nucleic acid sequence.

2. A process according to Claim 1, wherein said RNA first template comprises said specific nucleic acid sequence and wherein step (B) comprises providing single-stranded RNA in said reaction medium such that

(i) said first oligonucleotide primer hybridizes to said single-stranded RNA,

(ii) said RNA-directed DNA polymerase uses said single-stranded RNA as a template to synthesize a DNA second template by extension of said first oligonucleotide primer and thereby forms an RNA-DNA hybrid,

(iii) said ribonuclease hydrolyses RNA which comprises said RNA-DNA hybrid,

(iv) said second oligonucleotide primer hybridizes to said DNA second template,

(v) said DNA-directed DNA polymerase uses said second oligonucleotide primer as template to synthesize a functional promoter recognized by said RNA polymerase by extension of said DNA second template;

and

(vi) said DNA-directed RNA polymerase recognizes said functional promoter and transcribes said DNA second template, thereby providing copies of said RNA first template.

3. A process according to Claim 2, wherein step (B) comprises adding to said reaction medium said single-stranded RNA.

4. A process according to Claim 1, wherein said RNA first template comprises a sequence complementary to said specific nucleic acid sequence and wherein step (B) comprises providing single-stranded RNA in said reaction medium such that

(i) said second oligonucleotide primer hybridizes to said single-stranded RNA,

(ii) said RNA-directed DNA polymerase uses said RNA as a template to synthesize a complementary DNA by extension of said second oligonucleotide primer and thereby forms an RNA-DNA hybrid,

(iii) said ribonuclease hydrolyses RNA which comprises said RNA-DNA hybrid,

(iv) said first oligonucleotide primer hybridizes to said complementary DNA,

(v) said DNA-directed DNA polymerase uses said complementary DNA as template to synthesize said DNA second template and a functional promoter recognized by said RNA polymerase by extension of said first oligonucleotide primer;

and

(vi) said DNA-directed RNA polymerase recognizes said functional promoter and transcribes said DNA second template, thereby providing copies of said RNA first template.

5. A process according to Claim 4, wherein step (B) comprises adding to said reaction medium said single-stranded RNA.

6. A process according to Claim 1, wherein step (B) comprises adding to said reaction medium single-stranded DNA which comprises an antisense sequence of a promoter recognized by said RNA polymerase, such that

(i) said first oligonucleotide primer hybridizes to said single-stranded DNA,

(ii) said DNA-directed DNA polymerase uses said single-stranded DNA as template to synthesize said DNA second template and a functional promoter recognized by said RNA polymerase by extension of said first oligonucleotide primer;

and

(iii) said DNA-directed RNA polymerase recognizes said functional promoter and transcribes said DNA second template, thereby providing copies of said RNA first template.

7. A process according to Claim 6, wherein step (B) comprises adding to said reaction medium an RNA-DNA hybrid comprising said single-stranded DNA, such that said ribonuclease hydrolyzes RNA which comprises said RNA-DNA hybrid.

5

8. A process according to Claim 1, wherein step (B) comprises adding to said reaction medium single-stranded DNA which comprises said DNA second template, such that

10 (i) said second oligonucleotide primer hybridizes to said single-stranded DNA,

(ii) said DNA-directed DNA polymerase uses said second oligonucleotide primer as template to synthesize a functional promoter recognized by said RNA polymerase by extension of said DNA second template;

and

15 (iii) said DNA-directed RNA polymerase recognizes said functional promoter and transcribes said DNA second template, thereby providing copies of said RNA first template.

9. A process according to Claim 8, wherein step (B) comprises adding to said reaction medium an RNA-DNA hybrid comprising said single-stranded DNA, such that said ribonuclease hydrolyzes RNA which comprises said RNA-DNA hybrid.

20

10. A process according to Claim 2, wherein step (B) comprises adding to said reaction medium a DNA comprising a functional promoter recognized by said RNA polymerase, such that said DNA-directed RNA polymerase transcribes said DNA, thereby synthesizing said single-stranded RNA.

25

11. A process according to Claim 4, wherein step (B) comprises adding to said reaction medium a DNA comprising a functional promoter recognized by said RNA polymerase, such that said DNA-directed RNA polymerase transcribes said DNA, thereby synthesizing said single-stranded RNA.

12. A process according to Claim 1, wherein said second oligonucleotide primer further comprises an antisense sequence of a transcription initiation site for said DNA-directed RNA polymerase, said antisense sequence of said transcription initiation site being operatively linked to said antisense sequence of said promoter.

30

13. A process according to Claim 12, wherein said DNA-directed RNA polymerase is bacteriophage T7 RNA polymerase and wherein said antisense sequence of a transcription initiation site and said antisense sequence of said promoter together comprise the nucleotide sequence

AATTCTAATACGACTCACTATAGGGAG.

14. A process according to Claim 1, wherein step (B) further comprises adding a sample to said reaction medium under conditions such that, if said sample thereby provides RNA comprising an RNA first template which comprises said specific nucleic acid sequence or a sequence complementary to said specific nucleic acid sequence, said cycle ensues, and wherein said process further comprises, after step (C), a step (D) of monitoring said reaction medium for consumption of any of said reagents (i), (ii) and (vii) or for accumulation of any product of said cycle.

40

15. A process according to Claim 14, wherein step (D) comprises detecting a nucleic acid product of said cycle.

16. A process according to Claim 15, wherein step (D) comprises detecting said nucleic acid product using a nucleic acid probe.

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17. A process according to Claim 15, wherein step (D) comprises detecting said nucleic acid product using restriction endonucleases and electrophoretic separation.

55

18. A process according to Claim 15, wherein step (D) comprises monitoring the accumulation of said RNA first template.

19. A process according to Claim 15, wherein step (D) comprises monitoring the accumulation of said DNA second template.
20. A process according to Claim 15, wherein step (D) comprises monitoring DNA containing a functional promoter recognized by said RNA polymerase.
5
21. A process according to Claim 15, wherein step (D) comprises monitoring the accumulation of said RNA-DNA hybrid intermediate.
22. A process according to Claim 14, wherein step (D) further comprises comparing consumption of any reagent of said reagents (i), (ii) and (vii) or accumulation of any product of said cycle with a value representing consumption of said reagent or accumulation of said product in said reaction medium in the absence of said specific nucleic acid sequence and said sequence complementary thereto.
10
23. A process according to Claim 1, wherein said ribonuclease comprises *Escherichia coli* ribonuclease H.
15
24. A process according to Claim 1, wherein said ribonuclease comprises calf thymus ribonuclease H.
25. A process according to Claim 1, wherein said first oligonucleotide primer or said second oligonucleotide primer is bound reversibly to an immobilized support.
20
26. A process according to Claim 1, wherein said DNA-directed RNA polymerase is a bacteriophage RNA polymerase.
27. A process according to Claim 26, wherein said DNA-directed RNA polymerase is bacteriophage T7 RNA polymerase.
25
28. A process according to Claim 26, wherein said DNA-directed RNA polymerase is bacteriophage T3 polymerase.
29. A process according to Claim 26, wherein said DNA-directed RNA polymerase is bacteriophage ϕ II polymerase.
30
30. A process according to Claim 26, wherein said DNA-directed RNA polymerase is *Salmonella* bacteriophage sp6 polymerase.
35
31. A process according to Claim 26, wherein said DNA-directed RNA polymerase is *Pseudomonas* bacteriophage gh-1 polymerase.
32. A process according to Claim 1, wherein said RNA-directed DNA polymerase is a retrovirus reverse transcriptase.
40
33. A process according to Claim 32, wherein said retrovirus reverse transcriptase is avian myeloblastosis virus polymerase.
34. A process according to Claim 32, wherein said retrovirus reverse transcriptase is a Moloney murine leukemia virus polymerase.
45
35. A process according to Claim 1, wherein said DNA-directed DNA polymerase lacks exonuclease activity.
50
36. A process according to Claim 1, wherein all DNA polymerases in said reaction medium lack DNA exonuclease and endonuclease activity.
37. A process according to Claim 1, wherein said DNA-directed DNA polymerase is avian myeloblastosis virus polymerase.
55

38. A process according to Claim 1, wherein said DNA-directed DNA polymerase is DNA polymerase α or DNA polymerase β .

39. A process according to Claim 1, wherein said DNA-directed DNA polymerase is calf thymus DNA polymerase.

5 40. A process according to Claim 1, wherein step (C) comprises maintaining said conditions for a time between 30 minutes and 3 hours.

10 41. A kit for amplifying a specific nucleic acid sequence, comprising an assemblage of
(a) a receptacle containing a first oligonucleotide primer,
(b) a receptacle containing a second oligonucleotide primer comprising an antisense sequence of a promoter,
15 (c) a receptacle containing a ribonuclease that hydrolyses RNA of an RNA/DNA hybrid without hydrolyzing single- or double-stranded RNA or DNA,
(d) a receptacle containing an RNA-directed DNA polymerase,
(e) a receptacle containing a DNA-directed RNA polymerase,
(f) a receptacle containing a DNA-directed DNA polymerase,
20 (g) a receptacle containing ribonucleoside triphosphates and,
(h) a receptacle containing deoxyribonucleoside triphosphates.

Patentansprüche

1. Verfahren zur Amplifizierung einer spezifischen Nukleinsäuresequenz bei einer relativ konstanten Temperatur und ohne aufeinanderfolgende Zugabe von Reagenzien, umfassend die Schritte:
25 (A) Bereitstellung eines einzelnen Reaktionsmediums mit den Reagenzien enthaltend
(i) einen ersten Nukleotidprimer,
(ii) einen zweiten Nukleotidprimer, umfassend eine Antisense-Sequenz eines von einer RNA-Polymerase erkannten Promotors,
30 (iii) eine DNA-gerichtete RNA-Polymerase, die den Promotor erkennt,
(iv) eine RNA-gerichtete DNA-Polymerase,
(v) eine DNA-gerichtete DNA-Polymerase,
(vi) eine Ribonuklease, die RNA des RNA-DNA-Hybrids ohne Hydrolyse einzelsträngiger oder doppelsträngiger RNA oder DNA hydrolysiert, und
35 (vii) Ribonukleosid- und Deoxyribonukleosidtriphosphate;
anschließend
40 (B) Bereitstellung von RNA in einem ersten Reaktionsmedium enthaltend eine erste RNA-Matrize, die die spezifische Nukleinsäuresequenz oder eine zu dieser spezifischen Nukleinsäuresequenz komplementäre Sequenz enthält, unter solchen Bedingungen, so daß ein Zyklus gewährleistet ist, worin
(i) der erste Oligonukleotidprimer an die erste RNA-Matrize hybridisiert,
(ii) die RNA-gerichtete DNA-Polymerase die erste RNA-Matrize zur Synthesierung einer zweiten DNA-Matrize unter Verlängerung des ersten Oligonukleotidprimers verwendet, wobei ein RNA-DNA-Hybridintermediate gebildet wird,
45 (iii) die Ribonuklease RNA hydrolysiert, die das RNA-DNA-Hybridintermediat enthält,
(iv) der zweite Oligonukleotidprimer an die zweite DNA-Matrize hybridisiert,
(v) die DNA-gerichtete DNA-Polymerase den zweiten Oligonukleotidprimer als Matrize und zur Synthese eines funktionalen Promotors verwendet, der von der RNA-Polymerase erkannt werden kann, unter Verlängerung der zweiten DNA-Matrize; und
50 (vi) die DNA-gerichtete RNA-Polymerase den funktionalen Promotor erkennt und die zweite DNA-Matrize transkribiert, wodurch Kopien der ersten Matrizen RNA gebildet werden;
und anschließend
55 (C) Aufrechterhalten dieser Bedingungen für einen Zeitraum, der ausreichend ist, eine gewünschte Amplifizierung der spezifischen Nukleinsäuresequenz zu erreichen.

2. Verfahren nach Anspruch 1, worin die erste RNA-Matrize die spezifische Nukleinsäuresequenz enthält und worin Schritt (B) die Bereitstellung einzelsträngiger RNA in dem Reaktionsmedium umfaßt, so daß
(i) der erste Oligonukleotidprimer an die einzelsträngige RNA hybridisiert.

(ii) die RNA-gerichtete DNA-Polymerase die einzelsträngige RNA als Matrize zur Synthese einer zweiten DNA-Matrize durch Verlängerung des ersten Oligonukleotidprimers synthetisiert und hierdurch ein RNA-DNA-Hybrid erzeugt,

5 (iii) die Ribonuklease RNA, die das RNA-DNA-Hybrid enthält, hydrolysiert,

(iv) der zweite Oligonukleotidprimer an die zweite DNA-Matrize hybridisiert,

(v) die DNA-gerichtete DNA-Polymerase den zweiten Oligonukleotidprimer als Matrize zur Synthese eines funktionalen Promotors verwendet, der durch die RNA-Polymerase erkannt werden kann, unter Verlängerung der zweiten DNA-Matrize; und

10 (vi) die DNA-gerichtete RNA-Polymerase den funktionalen Promotor erkennt und die zweite DNA-Matrize transkribiert, wodurch Kopien der ersten RNA-Matrize erzeugt werden.

3. Verfahren nach Anspruch 2, worin Schritt (B) die Zugabe der einzelsträngigen RNA zum Reaktionsmedium umfaßt.

15 4. Verfahren nach Anspruch 1, worin die erste RNA-Matrize eine zur spezifischen Nukleinsäuresequenz komplementären Sequenz enthält, und worin Schritt (B) die Bereitstellung einer einzelsträngigen RNA im Reaktionsmedium umfaßt, so daß

(i) der zweite Oligonukleotidprimer an die einzelsträngige RNA hybridisiert,

(ii) die RNA-gerichtete DNA-Polymerase die RNA als Matrize zur Synthese einer komplementären 20 DNA durch Verlängerung des Seitenoligonukleotidprimers benutzt und dadurch ein RNA-DNA-Hybrid erzeugt,

(iii) die Ribonuklease RNA, die das RNA-DNA-Hybrid enthält, hydrolysiert,

(iv) der erste Oligonukleotidprimer an die komplementäre DNA hybridisiert,

(v) die DNA-gerichtete DNA-Polymerase die komplementäre DNA als Matrize zur Synthese der 25 zweiten DNA-Matrize und eines funktionalen Promotors, der durch die RNA-Polymerase erkannt wird, durch Verlängerung des ersten Oligonukleotidprimers verwendet und

(vi) die DNA-gerichtete RNA-Polymerase den funktionalen Promotor erkennt und die zweite DNA-Matrize transkribiert, wodurch Kopien der ersten RNA-Matrize zur Verfügung gestellt werden.

30 5. Verfahren nach Anspruch 4, worin Schritt (B) die Zugabe der einzelsträngigen RNA zum Reaktionsmedium umfaßt.

6. Verfahren nach Anspruch 1, worin Schritt (B) die Zugabe einzelsträngiger DNA, welche eine Antisense-Sequenz des durch die RNA-Polymerase erkannten Promotors enthält, umfaßt, so daß

35 (i) der erste Oligonukleotidprimer an die einzelsträngige DNA hybridisiert,

(ii) die DNA-gerichtete DNA-Polymerase die einzelsträngige DNA als Matrize unter Synthese der zweiten DNA-Matrize und eines funktionalen Promotors, der von der RNA-Polymerase erkannt wird, unter Verlängerung des ersten Oligonukleotidprimers verwendet,

und

40 (iii) die DNA-gerichtete RNA-Polymerase den funktionalen Promotor erkennt und die zweite DNA-Matrize transkribiert, wodurch Kopien der ersten RNA-Matrize zur Verfügung gestellt werden.

7. Verfahren nach Anspruch 6, worin Schritt (B) die Zugabe eines RNA-DNA-Hybrids, das einzelsträngige DNA enthält, zum Reaktionsmedium umfaßt, so daß die Ribonuklease die RNA, die das RNA-DNA-Hybrid enthält, hydrolysiert.

45 8. Verfahren nach Anspruch 1, worin Schritt (B) die Zugabe einzelsträngiger DNA, die die zweite DNA-Matrize enthält, umfaßt, so daß

(i) der zweite Oligonukleotidprimer an die einzelsträngige DNA hybridisiert,

(ii) die DNA-gerichtete DNA-Polymerase den zweiten Oligonukleotidprimer als Matrize unter Synthese eines funktionalen Promotors, der durch die RNA-Polymerase erkannt wird, unter Verlängerung 50 der zweiten DNA-Matrize verwendet;

und

(iii) die DNA-gerichtete RNA-Polymerase den funktionalen Promotor erkennt und die zweite DNA-Matrize transkribiert, wodurch Kopien der ersten RNA-Matrize zur Verfügung gestellt werden.

55 9. Verfahren nach Anspruch 8, worin Schritt (B) die Zugabe eines RNA-DNA-Hybrids, das die einzelsträngige DNA enthält, zum Reaktionsmedium umfaßt, so daß die Ribonuklease die RNA hydrolysiert,

welche das RNA-DNA-Hybrid enthält.

- 5 10. Verfahren nach Anspruch 2, worin Schritt (B) die Zugabe einer DNA, die einen von der RNA-Polymerase erkannten funktionalen Promotor enthält, zum Reaktionsmedium umfaßt, so daß die DNA-gerichtete RNA-Polymerase die DNA transkribiert, wodurch einzelsträngige RNA synthetisiert wird.
- 10 11. Verfahren nach Anspruch 4, worin Schritt (B) die Zugabe einer DNA, die einen durch die RNA-Polymerase erkannten funktionalen Promotor enthält, zum Reaktionsmedium umfaßt, so daß die DNA-gerichtete RNA-Polymerase die DNA transkribiert, wodurch die einzelsträngige RNA synthetisiert wird.
- 15 12. Verfahren nach Anspruch 1, worin der zweite Oligonukleotidprimer zusätzlich eine Antisense-Sequenz einer Transkriptionsinitierungsstelle für die DNA-gerichtete RNA-Polymerase enthält, wobei die Antisense-Sequenz der Transkriptionsinitierungsstelle operativ mit der Antisense-Sequenz des Promotors verknüpft ist.
- 20 13. Verfahren nach Anspruch 12, worin die DNA-gerichtete RNA-Polymerase Bakteriophage T7 RNA-Polymerase ist und worin die Antisense-Sequenz der Transkriptionsinitierungsstelle und die Antisense-Sequenz des Promotors zusammen die Nukleotidsequenz
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bilden.
- 25 14. Verfahren nach Anspruch 1, worin Schritt (B) ferner die Zugabe einer Probe zum Reaktionsmedium unter solchen Bedingungen umfaßt, daß, falls die Probe hierdurch RNA, die eine erste RNA-Matrice enthält, welche ihrerseits die spezifischen Nukleinsäuresequenz oder eine Sequenz komplementär zur spezifischen Nukleinsäuresequenz enthält, zur Verfügung steht, der Zyklus gewährleistet wird, und worin das Verfahren ferner nach Schritt (C) einen Schritt (D) umfaßt, in dem das Reaktionsmedium auf den Verbrauch einer der Reagenzien (i), (ii) und (vii) oder auf die Akkumulierung eines der Produkte des Zyklus überwacht wird.
- 30 15. Verfahren nach Anspruch 14, worin Schritt (D) den Nachweis eines Nukleinsäureprodukts des Zyklus umfaßt.
- 35 16. Verfahren nach Anspruch 15, worin Schritt (D) den Nachweis eines Nukleinsäureprodukts unter Verwendung einer Nukleinsäuresonde umfaßt.
17. Verfahren nach Anspruch 15, worin Schritt (D) den Nachweis eines Nukleinsäureprodukts unter Verwendung von Restriktionsendonukleasen und elektrophoretischer Auftrennung umfaßt.
- 40 18. Verfahren nach Anspruch 15, worin Schritt (D) die Überwachung der Akkumulierung der ersten RNA-Matrice umfaßt.
19. Verfahren nach Anspruch 15, worin Schritt (D) die Überwachung der Akkumulierung der zweiten DNA-Matrice umfaßt.
- 45 20. Verfahren nach Anspruch 15, worin Schritt (D) die Überwachung der DNA, die einen funktionalen Promotor, der durch die RNA-Polymerase erkannt wird, umfaßt.
21. Verfahren nach Anspruch 15, worin Schritt (D) die Überwachung der Akkumulierung des RNA-DNA-Hybridintermediats umfaßt.
- 50 22. Verfahren nach Anspruch 14, worin Schritt (D) zusätzlich den Vergleich des Verbrauchs von einem beliebigen der Reagenzien (i), (ii) und (vii) oder die Akkumulierung von einem der Produkte des Zyklus mit einem Wert, der den Verbrauch dieses Reagens oder die Akkumulation des Produkts im Reaktionsmedium in Abwesenheit der spezifischen Nukleinsäuresequenz und der hiergegen komplementären Sequenz umfaßt.
- 55 23. Verfahren nach Anspruch 1, worin die Ribonuklease Escherichia coli Ribonuklease H enthält.

24. Verfahren nach Anspruch 1, worin die Ribonuklease Kalbsthymus Ribonuklease H enthält.

25. Verfahren nach Anspruch 1, worin der erste Oligonukleotidprimer oder der zweite Oligonukleotidprimer reversibel an einen immobilisierten Träger gebunden ist.

5 26. Verfahren nach Anspruch 1, worin die DNA-gerichtete RNA-Polymerase eine Bakteriophage-RNA-Polymerase ist.

10 27. Verfahren nach Anspruch 26, worin die DNA-gerichtete RNA-Polymerase Bakteriophage T7-RNA-Polymerase ist.

28. Verfahren nach Anspruch 26, worin die DNA-gerichtete RNA-Polymerase Bakteriophage T3-Polymerase ist.

15 29. Verfahren nach Anspruch 26, worin die DNA-gerichtete RNA-Polymerase Bakteriophage ϕ II-Polymerase ist.

30. Verfahren nach Anspruch 26, worin die DNA-gerichtete RNA-Polymerase Salmonella-Bacteriophage sp6-Polymerase ist.

20 31. Verfahren nach Anspruch 26, worin die DNA-gerichtete RNA-Polymerase Pseudomonas-Bacteriophage gh-1-Polymerase ist.

32. Verfahren nach Anspruch 1, worin die RNA-gerichtete DNA-Polymerase eine reverse Transkriptase aus einem Retrovirus ist.

25 33. Verfahren nach Anspruch 32, worin die Retrovirus-reverse Transkriptase Vogel-Myeloblastosisvirus-Polymerase ist.

30 34. Verfahren nach Anspruch 32, worin die Retrovirus-reverse Transkriptase eine Moloney-Mäuseleukämievirus-Polymerase ist.

35 35. Verfahren nach Anspruch 1, worin die DNA-gerichtete DNA-Polymerase keine Exonukleaseaktivität besitzt.

36. Verfahren nach Anspruch 1, worin allen DNA-Polymerasen im Reaktionsmedium DNA-Exonuklease oder -Endonukleaseaktivität fehlt.

40 37. Verfahren nach Anspruch 1, worin die DNA-gerichtete DNA-Polymerase eine Vogel-Myeloblastosisvirus-Polymerase ist.

38. Verfahren nach Anspruch 1, worin die DNA-gerichtete DNA-Polymerase DNA-Polymerase alpha oder DNA-Polymerase beta ist.

45 39. Verfahren nach Anspruch 1, worin die DNA-gerichtete DNA-Polymerase Kalbsthymus DNA-Polymerase ist.

40 40. Verfahren nach Anspruch 1, worin Schritt (B) die Aufrechterhaltung dieser Bedingungen für einen Zeitraum von 30 min bis zu 3 h umfaßt.

50 41. Kit zur Amplifizierung einer spezifischen Nukleinsäuresequenz, umfassend die Anordnung aus einem
(a) Behälter, der einen ersten Oligonukleotidprimer enthält,
(b) einen Behälter, der einen zweiten Oligonukleotidprimer mit einer Antisense-Sequenz eines Promotors enthält,
55 (c) einen Behälter, der eine Ribonuklease enthält, die RNA eines eines RNA/DNA-Hybrids ohne Hydrolyse einzel- oder doppelsträngiger RNA oder DNA hydrolysiert,
(d) einen Behälter, der eine RNA-gerichtete DNA-Polymerase enthält,
(e) einen Behälter, der eine DNA-gerichtete RNA-Polymerase enthält,

- (f) einen Behälter, der eine DNA-gerichtete DNA-Polymerase enthält,
- (g) einen Behälter, der Ribonukleosidtriphosphate enthält und
- (h) einen Behälter, der Deoxyribonukleosidtriphosphate enthält.

5 **Revendications**

1. Procédé d'amplification d'une séquence d'acide nucléique particulière à une température relativement constante et sans addition en série de réactifs, comprenant les stades consistant à :
 - (A) réaliser un milieu réactionnel unique contenant des réactifs comprenant :
 - (i) une première amorce d'oligonucléotide,
 - (ii) une seconde amorce d'oligonucléotide comprenant une séquence antisens d'un promoteur reconnu par une ARN polymérase,
 - (iii) une ARN polymérase dirigée vers l'ADN qui reconnaît ce promoteur,
 - (iv) une ADN polymérase dirigée vers l'ARN,
 - (v) une ADN polymérase dirigée vers l'ADN,
 - (vi) une ribonucléase qui hydrolyse l'ARN d'un hybride ARN-ADN sans hydrolyser un ARN ou un ADN à brin unique ou à double brin, et
 - (vii) des ribonucléoside et désoxyribonucléoside triphosphates;
 - puis
 - (B) apporter dans ce milieu réactionnel un ARN comprenant un premier modèle d'ARN qui comprend cette séquence d'acide nucléique particulière ou une séquence complémentaire de cette séquence d'acide nucléique particulière dans des conditions telles qu'il en résulte un cycle dans lequel :
 - (i) cette première amorce d'oligonucléotide s'hybride à ce premier modèle d'ARN,
 - (ii) cette ADN polymérase dirigée vers l'ARN utilise ce premier modèle d'ARN pour synthétiser un second modèle d'ADN par allongement de cette première amorce d'oligonucléotide et forme ainsi un intermédiaire d'hybride ARN-ADN,
 - (iii) cette ribonucléase hydrolyse l'ARN qui comprend cet intermédiaire d'hybride ARN-ADN,
 - (iv) cette seconde amorce d'oligonucléotide s'hybride à ce second modèle d'ADN,
 - (v) cette ADN polymérase dirigée vers l'ADN utilise cette seconde amorce d'oligonucléotide comme modèle pour synthétiser un promoteur fonctionnel reconnu par cette ARN polymérase par allongement de ce second modèle d'ADN; et
 - (vi) cette ARN polymérase dirigée vers l'ADN reconnaît ce promoteur fonctionnel et transcrit ce second modèle d'ADN, réalisant ainsi des copies de ce premier modèle d'ARN;
 - puis
 - (C) maintenir ces conditions pendant un temps suffisant pour réaliser l'amplification désirée de cette séquence d'acide nucléique particulière.
2. Procédé selon la revendication 1, dans lequel ce premier modèle d'ARN comprend cette séquence d'acide nucléique particulière et dans lequel le stade (B) comprend le fait d'apporter un ARN à brin unique dans ce milieu réactionnel, tel que :
 - (i) cette première amorce d'oligonucléotide s'hybride à cet ARN à brin unique,
 - (ii) cette ADN polymérase dirigée vers l'ARN utilise cet ARN à brin unique comme modèle pour synthétiser un second modèle d'ADN par allongement de cette première amorce d'oligonucléotide, et forme ainsi un hybride ARN-ADN,
 - (iii) cette ribonucléase hydrolyse l'ARN qui comprend cet hybride ARN-ADN,
 - (iv) cette seconde amorce d'oligonucléotide s'hybride à ce second modèle d'ADN,
 - (v) cette ADN polymérase dirigée vers l'ADN utilise cette seconde amorce d'oligonucléotide comme modèle pour synthétiser un promoteur fonctionnel reconnu par cette ARN polymérase par allongement de ce second modèle d'ADN; et
 - (vi) cette ARN polymérase dirigée vers l'ADN reconnaît ce promoteur fonctionnel et transcrit ce second modèle d'ADN, réalisant ainsi des copies de ce premier modèle d'ARN.
3. Procédé selon la revendication 2, dans lequel le stade (B) comprend l'addition à ce milieu réactionnel de cet ARN à brin unique.
4. Procédé selon la revendication 1, dans lequel ce premier modèle d'ARN comprend une séquence complémentaire de cette séquence d'acide nucléique particulière et dans lequel le stade (B) comprend

le fait d'apporter un ARN à brin unique dans ce milieu réactionnel, tel que :

- (i) cette seconde amorce d'oligonucléotide s'hybride à cet ARN à brin unique,
- (ii) cette ADN polymérase dirigée vers l'ARN utilise cet ARN comme modèle pour synthétiser un ADN complémentaire par allongement de cette seconde amorce d'oligonucléotide, et forme ainsi un hybride ARN-ADN,
- (iii) cette ribonucléase hydrolyse l'ARN qui comprend cet hybride ARN-ADN,
- (iv) cette première amorce d'oligonucléotide s'hybride à cet ADN complémentaire,
- (v) cette ADN polymérase dirigée vers l'ADN utilise cet ADN complémentaire comme modèle pour synthétiser ce second modèle d'ADN et un promoteur fonctionnel reconnu par cette ARN polymérase par allongement de cette première amorce d'oligonucléotide; et
- (vi) cette ARN polymérase dirigée vers l'ADN reconnaît ce promoteur fonctionnel et transcrit ce second modèle d'ADN, réalisant ainsi des copies de ce premier modèle d'ARN.

5. Procédé selon la revendication 4, dans lequel le stade (B) comprend l'addition à ce milieu réactionnel de cet ARN à brin unique.
10. Procédé selon la revendication 1, dans lequel le stade (B) comprend l'addition à ce milieu réactionnel d'un ADN à brin unique qui comprend une séquence antisens d'un promoteur reconnu par cette ARN polymérase, tel que :
 - (i) cette première amorce d'oligonucléotide s'hybride à cet ADN à brin unique,
 - (ii) cette ADN polymérase dirigée vers l'ADN utilise cet ADN à brin unique comme modèle pour synthétiser ce second modèle d'ADN et un promoteur fonctionnel reconnu par cette ARN polymérase par allongement de cette première amorce d'oligonucléotide; et
 - (iii) cette ARN polymérase dirigée vers l'ADN reconnaît ce promoteur fonctionnel et transcrit ce second modèle d'ADN, réalisant ainsi des copies de ce premier modèle d'ARN.
15. Procédé selon la revendication 6, dans lequel le stade (B) comprend l'addition à ce milieu réactionnel d'un hybride ARN-ADN comprenant cet ADN à brin unique, tel que cette ribonucléase hydrolyse l'ARN qui comprend cet hybride ARN-ADN.
20. Procédé selon la revendication 1, dans lequel le stade (B) comprend l'addition à ce milieu réactionnel d'un ADN à brin unique qui comprend ce second modèle d'ADN, tel que :
 - (i) cette seconde amorce d'oligonucléotide s'hybride à cet ADN à brin unique,
 - (ii) cette ADN polymérase dirigée vers l'ADN utilise cette seconde amorce d'oligonucléotide comme modèle pour synthétiser un promoteur fonctionnel reconnu par cette ARN polymérase par allongement de ce second modèle d'ADN; et
 - (iii) cette ARN polymérase dirigée vers l'ADN reconnaît ce promoteur fonctionnel et transcrit ce second modèle d'ADN, réalisant ainsi des copies de ce premier modèle d'ARN.
25. Procédé selon la revendication 8, dans lequel le stade (B) comprend l'addition à ce milieu réactionnel d'un hybride ARN-ADN comprenant cet ADN à brin unique, tel que cette ribonucléase hydrolyse l'ARN qui comprend cet hybride ARN-ADN.
30. Procédé selon la revendication 2, dans lequel le stade (B) comprend l'addition à ce milieu réactionnel d'un ADN comprenant un promoteur fonctionnel reconnu par cette ARN polymérase, tel que cette ARN polymérase dirigée vers l'ADN transcrive cet ADN, synthétisant ainsi cet ARN à brin unique.
35. Procédé selon la revendication 4, dans lequel le stade (B) comprend l'addition à ce milieu réactionnel d'un ADN comprenant un promoteur fonctionnel reconnu par cette ARN polymérase, tel que cette ARN polymérase dirigée vers l'ADN transcrive cet ADN, synthétisant ainsi cet ARN à brin unique.
40. Procédé selon la revendication 8, dans lequel le stade (B) comprend l'addition à ce milieu réactionnel d'un hybride ARN-ADN comprenant cet ADN à brin unique, tel que cette ribonucléase hydrolyse l'ARN qui comprend cet hybride ARN-ADN.
45. Procédé selon la revendication 11, dans lequel cette seconde amorce d'oligonucléotide comprend en outre une séquence antisens d'un site d'initiation de la transcription pour cette ARN polymérase dirigée vers l'ADN, cette séquence antisens de ce site d'initiation de la transcription étant liée dans son fonctionnement à cette séquence antisens de ce promoteur.
50. Procédé selon la revendication 1, dans lequel cette seconde amorce d'oligonucléotide comprend en outre une séquence antisens d'un site d'initiation de la transcription pour cette ARN polymérase dirigée vers l'ADN, cette séquence antisens de ce site d'initiation de la transcription étant liée dans son fonctionnement à cette séquence antisens de ce promoteur.
55. Procédé selon la revendication 1, dans lequel cette seconde amorce d'oligonucléotide comprend en outre une séquence antisens d'un site d'initiation de la transcription pour cette ARN polymérase dirigée vers l'ADN, cette séquence antisens de ce site d'initiation de la transcription étant liée dans son fonctionnement à cette séquence antisens de ce promoteur.

13. Procédé selon la revendication 12, dans lequel cette ARN polymérase dirigée vers l'ADN est l'ARN polymérase du bactériophage T7 et dans lequel cette séquence antisens d'un site d'initiation de la transcription et cette séquence antisens de ce promoteur comprennent ensemble la séquence de nucléotide.

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14. Procédé selon la revendication 1, dans lequel le stade (B) comprend en outre l'addition d'un échantillon à ce milieu réactionnel dans des conditions telles que, si cet échantillon fournit ainsi un ARN comprenant un premier modèle d'ARN qui comporte cette séquence particulière d'acide nucléique ou une séquence complémentaire de cette séquence particulière d'acide nucléique, il en résulte ledit cycle, et dans lequel ce procédé comprend en outre, après le stade (C), un stade (D) de contrôle de ce milieu réactionnel en ce qui concerne la consommation de n'importe quel réactif parmi ces réactifs (i), (ii) et (vii) ou l'accumulation de n'importe quel produit de ce cycle.

15. Procédé selon la revendication 14, dans lequel le stade (D) comprend la détection d'un acide nucléique formé dans ce cycle.

16. Procédé selon la revendication 15, dans lequel le stade (D) comprend la détection de cet acide nucléique formé en utilisant une sonde d'acide nucléique.

20 17. Procédé selon la revendication 15, dans lequel le stade (D) comprend la détection de cet acide nucléique formé en utilisant des endonucléases de restriction et une séparation électrophorétique.

25 18. Procédé selon la revendication 15, dans lequel le stade (D) comprend le contrôle de l'accumulation de ce premier modèle d'ARN.

19. Procédé selon la revendication 15, dans lequel le stade (D) comprend le contrôle de l'accumulation de ce second modèle d'ADN.

30 20. Procédé selon la revendication 15, dans lequel le stade (D) comprend le contrôle de l'ADN contenant un promoteur fonctionnel reconnu par cette ARN polymérase.

21. Procédé selon la revendication 15, dans lequel le stade (D) comprend le contrôle de l'accumulation de cet intermédiaire d'hybride ARN-ADN.

35 22. Procédé selon la revendication 14, dans lequel le stade (B) comprend en outre le fait de comparer la consommation de n'importe quel réactif parmi ces réactifs (i), (ii) et (vii) ou l'accumulation de n'importe quel produit de ce cycle à une valeur représentant la consommation de ce réactif ou l'accumulation de ce produit dans ce milieu réactionnel en l'absence de cette séquence particulière d'acide nucléique et de la séquence qui lui est complémentaire.

40 23. Procédé selon la revendication 1, dans lequel cette ribonucléase comprend la ribonucléase H d'*Escherichia coli*.

45 24. Procédé selon la revendication 1, dans lequel cette ribonucléase comprend de la ribonucléase H de thymus de veau.

25. Procédé selon la revendication 1, dans lequel cette première amorce d'oligonucléotide ou cette seconde amorce d'oligonucléotide est liée de manière réversible à un support immobilisé.

50 26. Procédé selon la revendication 1, dans lequel cette ARN polymérase dirigée vers l'ADN est de l'ARN polymérase de bactériophage.

55 27. Procédé selon la revendication 26, dans lequel cette ARN polymérase dirigée vers l'ADN est de l'ARN polymérase de bactériophage T7.

28. Procédé selon la revendication 26, dans lequel cette ARN polymérase dirigée vers l'ADN est de la polymérase de bactériophage T3.

29. Procédé selon la revendication 26, dans lequel cette ARN polymérase dirigée vers l'ADN est de la polymérase de bactériophage ϕ II.

5 30. Procédé selon la revendication 26, dans lequel cette ARN polymérase dirigée vers l'ADN est de la polymérase de bactériophage sp6 de *Salmonella*.

10 31. Procédé selon la revendication 26, dans lequel cette ARN polymérase dirigée vers l'ADN est de la polymérase de bactériophage gh-1 de *Pseudomonas*.

15 32. Procédé selon la revendication 1, dans lequel cette ADN polymérase dirigée vers l'ARN est une transcriptase inverse de rétrovirus.

20 33. Procédé selon la revendication 32, dans lequel cette transcriptase inverse de rétrovirus est une polymérase du virus de la myéloblastose aviaire.

25 34. Procédé selon la revendication 32, dans lequel cette transcriptase inverse de rétrovirus est une polymérase de virus de la leucémie murine Moloney.

30 35. Procédé selon la revendication 1, dans lequel cette ADN polymérase dirigée vers l'ADN est dépourvue d'activité d'exonucléase.

36. Procédé selon la revendication 1, dans lequel toutes les ADN polymérasées de ce milieu réactionnel sont dépourvues d'activité d'ADN exonucléase et endonucléase.

35 37. Procédé selon la revendication 1, dans lequel cette ADN polymérase dirigée vers l'ADN est une polymérase de virus de la myéloblastose aviaire.

40 38. Procédé selon la revendication 1, dans lequel cette ADN polymérase dirigée vers l'ADN est une ADN polymérase α ou une ADN polymérase β .

45 39. Procédé selon la revendication 1, dans lequel cette ADN polymérase dirigée vers l'ADN est une polymérase d'ADN de thymus de veau.

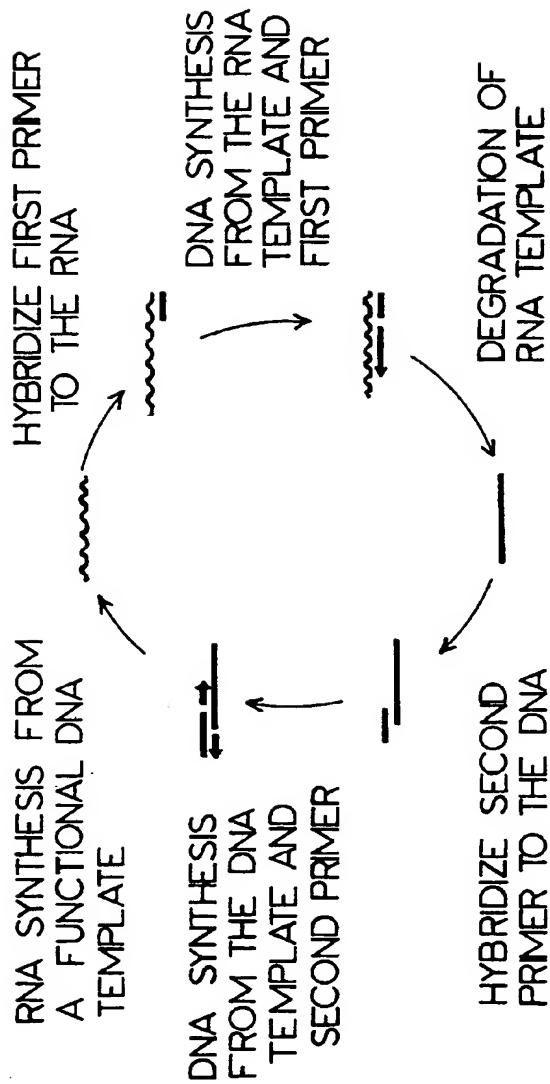
40 40. Procédé selon la revendication 1, dans lequel le stade (C) comprend le maintien de ces conditions pendant une durée comprise entre 30 minutes et 3 heures.

45 41. Nécessaire pour amplifier une séquence d'acide nucléique particulière comprenant l'assemblage :
(a) d'un réceptacle contenant une première amorce d'oligonucléotide,
(b) d'un réceptacle contenant une seconde amorce d'oligonucléotide comprenant une séquence antisens d'un promoteur,
(c) un réceptacle contenant une ribonucléase qui hydrolyse l'ARN d'un hybride ARN-ADN sans hydrolyser l'ARN ou l'ADN à brin unique ou à double brin,
(d) un réceptacle contenant une ADN polymérase dirigée vers l'ARN,
(e) un réceptacle contenant une ARN polymérase dirigée vers l'ADN,
(f) un réceptacle contenant une ADN polymérase dirigée vers l'ADN,
(g) un réceptacle contenant des ribonucléoside triphosphates et,
(h) un réceptacle contenant des désoxyribonucléoside triphosphates.

50

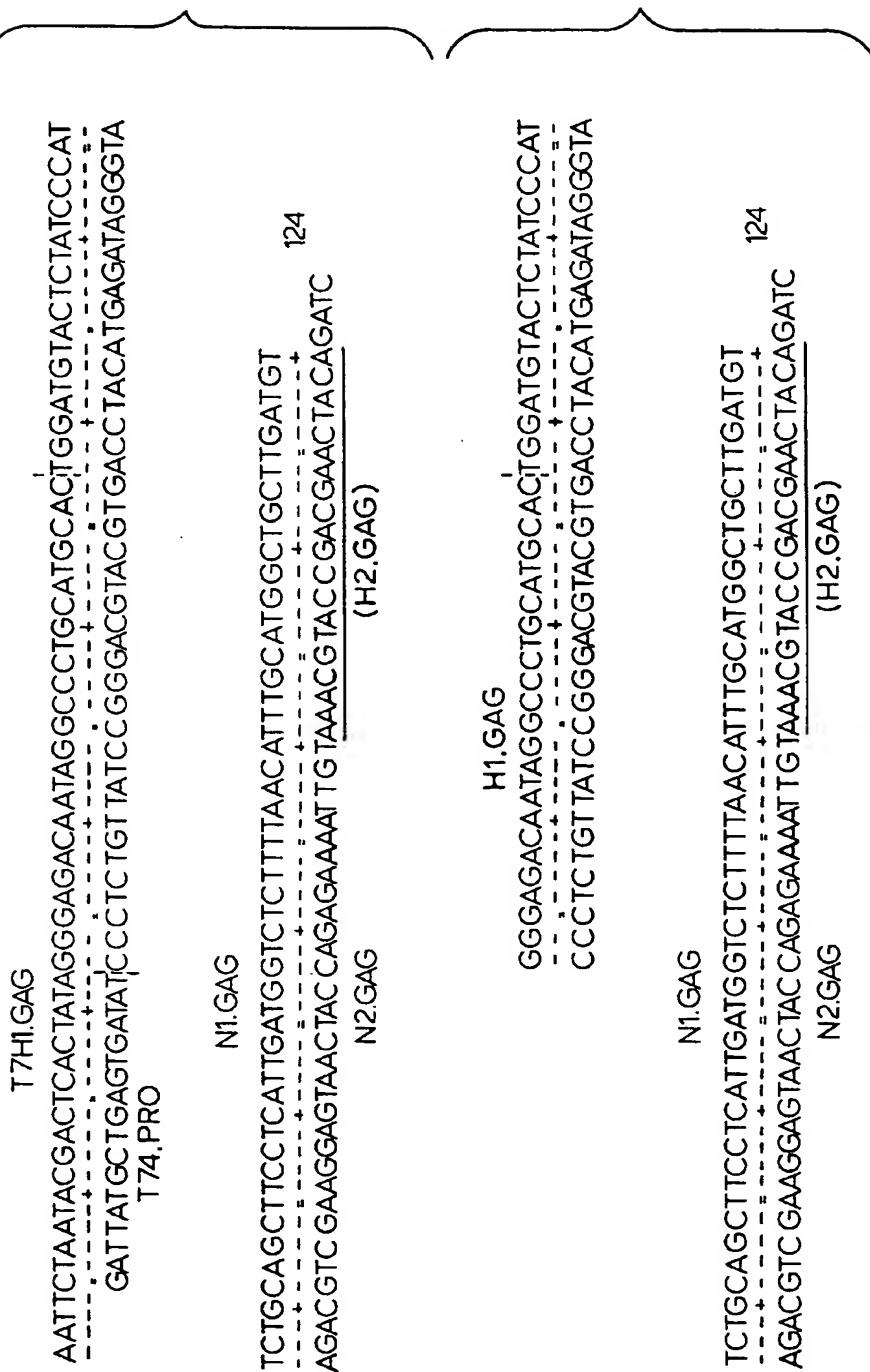
55

FIG. 1.



A

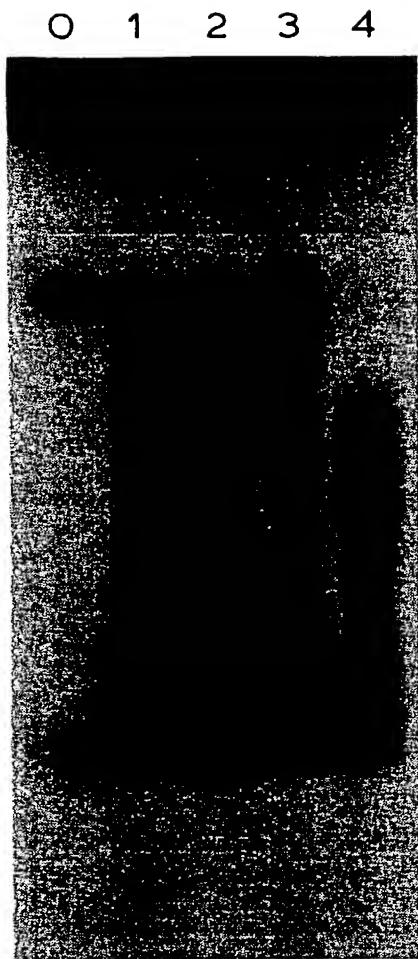
FIG.2.



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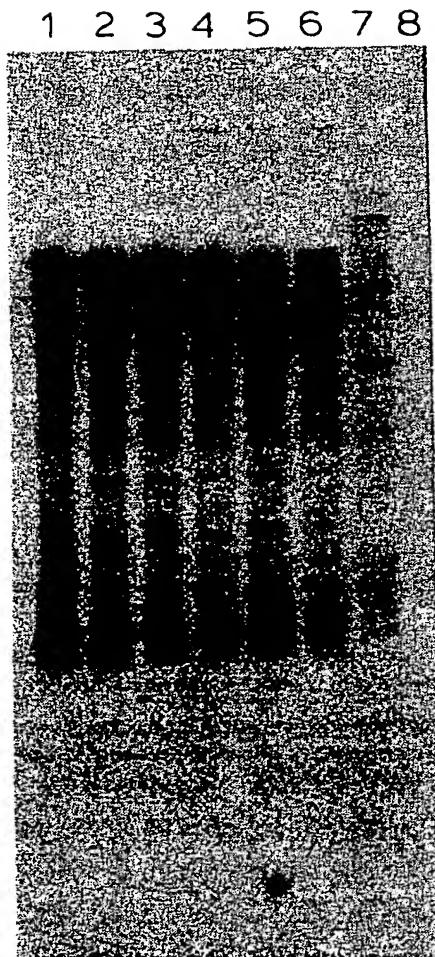
FIG.3.



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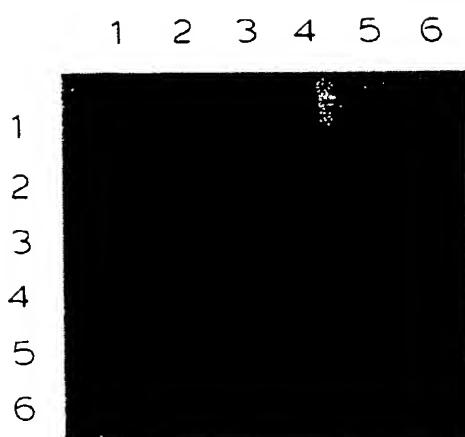
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FIG.4.



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FIG.5.



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FIG. 6.

0 1 2 3 4 5 6 11 12

